A modified *paperfuge* technique in evaluating anemia in phenylhydydrazine-induced ICR

Deepakchandran Koutharapu[1], Johanne P. Nuevarez[2], and Adrian P. Ybañez[3]

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ABSTRACT

Anemia is a common observation in several diseases in humans and animals. This condition is evaluated through the blood using parameters, including the packed cell volume (PCV). However, quick diagnosis might take longer, especially in resource limited areas, as blood samples still need to be processed in the laboratory. In this experimental study, a modified *paperfuge* technique was evaluated to assess anemia in an animal model. A total of 16 ICR mice were used. After animal acclimatization for ten days, persistent anemia (hemolytic) was induced in nine mice by administering phenylhydrazine every other day for three times. Only distilled water was used in the control mice. After seven days, blood was extracted from the mice. Complete blood count using a species specific hematology machine was performed. PCV was further evaluated using the centrifuge machine and the modified *paperfuge* technique. Results using the hematology machine showed that the phenylhydrazine-induced mice had lower red blood cell count ($\bar{x}$= 4.26 x 10^6/µL, SD=0.93) and PCV ($\bar{x}$=32.5, SD=7.5) than the red blood cell count ($\bar{x}$=5.26 x 10^6/µL, SD=1.1) and PCV ($\bar{x}$=36.5, SD=5.8) of the control mice. PCV readings from the three methods were strongly correlated with the RBC count. On the other hand, PCV readings from the paperfuge technique were also strongly and significantly correlated with the centrifuge (R=0.99, p=0.000) and hematology machine (R=0.89, p=0.000) results. Further analysis revealed that there was no significant difference observed between the PCV readings of the CBC machine, the centrifuge and the modified *paperfuge* (treatment group: p=0.39, control group: p=0.09). Results suggest that the modified *paperfuge* technique can be reliable in measuring PCV to evaluate anemia. Further studies are recommended to compare results in anemic animals and humans.

Keywords: anemia, mice, paperfuge, PCV, phenylhydrazine

I. Introduction

Complete blood count is a routine procedure performed in the assessment of heath and disease (Grimm, Neaton, & Ludwig, 1985; Rosman, 2003; Ybañez et al., 2016). Among the parameters include the hematocrit (HCT) or the packed cell volume (PCV). PCV is the volume percentage (vol%) of red blood cells in the blood (Purves, Sadava, Orians, & Craig 2004). PCV readings can give information on the health status of an individual, most especially when anemia is a feature of the suspected disease. Anemia refers to the decrease in the total amount of red blood cells (RBCs) or hemoglobin in the blood (Valentine & Paglia, 1984), which can also be reflected in the PCV. PCV is often used to indirectly estimate the RBC counts (Uthman, 2009). PCV has been used for evaluating dengue fever (Kularatne, Gawarammana, & Kumarasiri, 2005) and malaria (Davis et al., 1990), and for suitability of blood samples for blood transfusion (Goodnough, Breeher, Kanter, & AuBuchon, 1999).

PCV is evaluated using different methods. The gold standard of measuring PCV is by centrifugation (Myers & Browne, 2007). With modern laboratory equipment, PCV is not directly measured but rather calculated based on RBC indices. It has been shown that automated machines may yield different results than the measured PCV using the gold standard (Gebretsadkan, Ambachew, & Birhaneselassie, 2015). However, these two methods require equipment which can be expensive, or will require more time as samples would need to be transported to a laboratory. Hence, measurement of PCV can be difficult in the field.

Recently, an ultralow-cost, hand-powered paper-centrifuge called *paperfuge* was invented by researchers from Stanford University. It was inspired by the mechanics of a whirligig toy. The paperfuge can reach speeds of 125,000 rpm, and can separate pure plasma from whole blood in less than 1.5 minutes, and isolate malaria parasites in 15 minutes. This method can open up opportunities for point-of-care diagnostics in resource-poor settings (Bhamla et al., 2017).

In the Philippines, determining PCV is usually

accomplished using a centrifuge or a hematology machine as part of the routine parameters. Evaluation on the application of paperfuge has not been performed in the country. Its potential use is important as there are many areas with low diagnostic resource settings. Studies that would evaluate the applicability of paperfuge is therefore necessary. Moreover, the use of paperfuge in evaluating induced-anemia in animal models has not been performed yet. Thus, this study was conducted.

II. METHODOLOGY

This study was an experimental type, employing a completely randomized design. The study evaluated the differences of the hematocrit values between paperfuge technique, centrifuge machine and hematology machine (Mindray BC-5000 Vet) in ICR mice.

A total of 16 male ICR mice (12-16 weeks old weighing 22-37 grams) that were sourced from the University of San Carlos Animal Laboratory Facility were used. The mice were randomly divided into two: group 1 (phenylhydrazine-induced) with 9 mice, and group 2 (control-normal saline solution-NSS) with 7 mice. Animal experiment was conducted at the UV-Gullas College of Medicine Laboratory Animal Facility, a Philippine Association for Laboratory Animal Science (PALAS)-accredited facility. Centrifugation was performed at the laboratory of Vicente Gullas Memorial Hospital, Banilad, Mandaue City while samples for complete blood count was sent out to the GPY Veterinare Animale Veterinary Clinic, Punta Princesa, Tres de Abril, Cebu City.

The following were used: boxes with feeders and drinkers for housing mice, syringes for drug injection,
digital weighing scale for the mice, paperfuge and centrifuge machine. Paperfuge was made from the cardboard and strings. Phenylhydrazine was obtained from the Department of Research, Gullas College of Medicine, University of the Visayas.

Animal marking and assignment to groups and cages. Upon arrival at the animal facility, the animals were randomly assigned to cages and marked at the base of the tail using permanent markers for identification. Cages were also randomly assigned to the different treatment groups. Health of animals was assessed by physical examination.

Animal acclimatization, monitoring and maintenance. The mice were acclimatized for ten days. Commercial feeds and water were provided ad libitum. Lighting was on a 12 hour dark-light cycle, and ambient temperature was maintained between 24 to 26 degree Celsius. Beddings were changed every three days. Body score, appearance and attitude (Foltz & Ullma-Cullere 1999; Bekkevold, Robertson, Reinhand, Battles, & Rowland, 2013) were assessed daily. Body weight was monitored daily.

Administration of phenylhydrazine (PHZ) and NSS Normal Saline Solution (NSS). PHZ was administered via the intraperitoneal (IP) route at a dose of 0.1ml per 30 grams. On the other hand, the same dose of NSS was also administered on the control group by the IP route. This procedure was performed every other day for three times.

Anesthetization, blood collection and euthanasia. Mice were anesthetized using Zoletil 50 (tiletamine/zolazepam) (50 mg/ml) at a dose of 0.2 ml per 30 grams of body weight of mice. Blood (0.5 ml) was aseptically collected via facial vein or intracardiac route and placed in EDTA microtainer. After collection, mice were euthanized.

Creation of paperfuge. Paperfuge was initially made using cardboard guided by the specifications of Bhamla et al. (2016). However, initial trial use of the paperfuge was not successful due to different sizes of the available hematocrit tubes. Thus, the size was modified accordingly. Also, instead of pulling it sidewards, paperfuge was pulled upwards to imitate the parallel centrifugation of movement of centrifuge machines. The technique was performed for 10 minutes.

Blood from EDTA tube was transferred to 2 sets of microhematocrit capillary tubes: one for the centrifuge machine and the other for paperfuge. The remaining blood from EDTA tube was sent out for CBC at GPY Veterinare Animale Animal Clinic, Cebu City, Philippine. Results of the paperfuge and centrifuge machine were measured using a hematocrit scale.

Centrifuge technique. A small amount of blood (about 0.05 to 0.1ml) was drawn into a thin microcapillary tube and was sealed with wax and clay. The tube was then placed in the centrifuge. Centrifugation was performed for five minutes. The red cells settle at the bottom and form a red column and were separated from the straw-colored serum column by a small area composed of white blood cells. PCV reading is obtained by the height of the red cell column divided by the height of the total fluid in the capillary tube. The PCV level in the microhematocrit capillary tube gave the value of the hematocrit using the hematocrit measuring scale (Stockham & Scott, 2013).

Data processing and analysis. Results were recorded in tally sheets and encoded in Microsoft Excel. Encoded data was imported to a statistical software. Body weight and data from the complete blood count were compared between groups using independent t-test. For the body score, appearance score and attitude score, Kruskal Wallis was used. Significance level was set at 5%.

Hematocrit values from the paperfuge, centrifuge machine and CBC machine were compared and analyzed using one-way analysis of variance (ANOVA). Correlation between obtained hematocrit values and with RBC counts were assessed using simple linear regression.

The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act of the Philippines (RA 8485) and AO 45 of the Bureau of the Animal Industry. Study protocol was approved by the Institutional Animal Care and Use Committee of the University of the Visayas-Gullas College of Medicine, Inc.

III. RESULTS AND DISCUSSION

Observations on the body score, appearance, attitude score and body weight of experimental animals were similar, suggesting that administration of PHZ may cause no readily observable signs that can be manifested within seven days. Evaluating grossly using observed signs may not be totally reliable. On the other hand, complete blood count revealed that the RBC and hemoglobin count of the treatment groups were lower than the control group (Table 1), which supported the effect of PHZ to induce anemia in mice (Hara & Ogawa, 1976; Spivak, Toretti, & Dickerman, 1973; Paul et al., 1999). The treatment also had higher WBC counts, which may imply higher stress level due to the PHZ administration (Abramson & Melton, 2000).

Table 1
Complete blood count values of ICR mice obtained using Hematology machine (mindray)

Parameters	Unit	Control	Treatment
White blood cell count	x 10^3	5.38	7.45
Red Blood Cell count	x 10^6	5.26	4.26
Hemoglobin	g/dl	120.43	99.67
Packed Cell Volume	%	36.5	32.54
Platelet count	x 10^3	425.14	478.71
Differential count			
Basophil	%	0.54	1.08
Eosinophil	%	4.23	6.26
Lymphocytes	%	62.39	48.07
Neutrophils	%	26.77	38.98

PCV from treatment groups appeared to have lower readings than the control (Table 2). This indicated that PHZ was successful in inducing anemia in the animals. Comparing the PCV readings from the three different techniques, results showed that the paperfuge technique appeared to be higher than the centrifuge and hematology machine. Although Bhamla et al. (2017) mentioned that it will only take 1.5 minutes to separate the blood compartments in the microcapillary tube, the duration in the present experiment was ten minutes. Optimization of the method can be explored to determine the optimal time duration needed to effectively separate the blood compartments, most especially that the materials used at the local setting can be different.

Table 2
PCV readings of phenylhydrazine-induced and control (NSS) ICR mice using the paperfuge, centrifuge and hematology machine

Group	Paperfuge		Centrifuge machine		Hematology machine (Mindray BC-5000 Vet)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Treatment (n=9)	37.78	9.26	33.78	7.93	32.54	7.47
Control (n=7)	43.00	5.69	37.00	5.92	36.50	5.81

Statistical analysis revealed no significant differences on the bodyweight, body score, appearance, and attitude score (data not shown). On the other hand, analysis revealed that RBC counts were strongly and highly correlated with the PCV values from the three different techniques (Table 2). This indicates that PCV can be a good predictor of the RBC count using either of the three methods. Further analysis also revealed that the PCV readings from the three methods were found to have no significant differences. It implies that even at ten minutes of spinning, results can already be comparable to other methods, and that the paperfuge technique can be a substitute to the centrifuge and hematology machine. The results of this study corroborated with the findings of Bhamla et al. (2017).

Table 3
Results of statistical analyses

Simple linear regression analysis

Comparison	r	r^2	Equation
RBC count and centrifuge technique	0.835**	0.696	RBC= 0.555 + 0.114 (PCV)
Hematology machine	0.835**	0.897	RBC= -0.028 + 0.135 (PCV)
Paperfuge	0.835**	0.714	RBC= 0.623 + 0.099 (PCV)
Paperfuge and Centrifuge technique	0.988**	0.975	PCV (centrifuge)=1.428 + 0.85(PCV-paperfuge)
Paperfuge and Hematology machine	0.889**	0.79	PCV (hematology machine)=3.455 + 0.929(PCV-paperfuge)

Analysis of variance (ANOVA)

Parameter	df	F	Interpretation
PCV values - treatment group	2	0.989	No significant difference
PCV values - control group	2	2.718	No significant difference

*** Significant at 0.01 level*

Paperfuge is a promising technology that can be easily utilized by many human and animal health practitioners. This technology is most applicable to areas of low resource settings. However, optimiszation of this method and its application to several other disase for diagnosis remains a challenge. Futher studies maybe

conducted in the Philippines utilizing locally and readily available materials to create the paperfuge.

IV. CONCLUSION

The modified paperfuge technique can be a reliable method in evaluating PCV in anemia-induced and normal mice. Further studies to optimize its condition must be explored. The design of the paperfuge may be modified to conform to the existing locally available materials. Also, its application in actual disease conditions in the field can also be studied.

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AUTHORS

Koutharapu, Deepak chandran who is currently a student of Gullas College of Medicine, Banilad, Cebu, Philippines was born on May 20, 1993, in Andhra Pradesh, India; Completed his pre medicine course BS biology in Cebu Doctor's University ,Cebu, Philippines and graduated on 2014;completed 10th class in Montessori Public School, in Tenali, Andhra Pradesh, India and graduated on 2010. Completed 11th and 12th in Sri Chaitanya educational institutions, Vijayawada, Andhra Pradesh, India and graduated on 2012.

He will be graduating from Gullas College of Medicine on 2019 and is planning to pursue his specialization in U. S. A.

Johanne P. Nuevarez was born in the Province of Camiguin, a politically part of Northern Mindanao Region on April 18, 1984. Having been raised in Cebu City, she finished the degree of Doctor of Veterinary Medicine at Southwestern University on May 2007. And is currently working as a Laboratory and Research Associate at the University of the Visayas-Gullas College of Medicine, Laboratory Animal Facilty. She also works part-time as an Associate Veterinarian at GPY Veterinary Animale Veterinary Clinic. And is looking forward to finish her Masters Degree on Public Health in October of 2018.

Adrian P. Ybañez, BAS, DVM, MBA, PhD, Dip. PCCP (Cand), Dip. PCVPH (Cand) is the Proffessor II, Department of Research and Laboratory Animal Facility, Gullas College of Medicine, Inc. and Research Consultant for the Laboratory Animal Research/Facility, University of San Carlos and University of Southern Philippines Foundation.

He is awarded as grand winner of the Outstanding Cebuano Youth Leader (TOCYL) – young professional category by the Cebu City government , Most Outstanding Veterinarian in the Veterinary Research Practice by the Veterinary Practitioners Association of the Philippines, the Outstanding Cebuano Awardee (Science and Technology Field) and Oustanding Young Professional in Veterinary Research by the Philippine Society of Animal Science.